Characterization of bacteriocin produced by *Lactobacillus brevis* isolated from traditional fermented tomatoes juice

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Abstract: Biopreservation is the use of natural or controlled microbiota or antimicrobials as a way of preserving food and extending its shelf life. The biopreservation of food, especially utilizing lactic acid bacteria (LAB) that are inhibitory to food spoilage microbes, has been practiced since early ages, at first unconsciously but eventually with an increasingly robust scientific foundation. Beneficial bacteria or the fermentation products produced by these bacteria are used in biopreservation to control spoilage and render pathogens inactive in food. The purpose of this present work was to evaluate the efficacy of 65 strains isolated from fermented tomato juice under anaerobic conditions as biocontrol agents against fungi and the food spoilage bacteria. The antagonistic activity of isolated strains was tested *in vitro* using the well diffusion assay and the spot agar method. ISOL03 showed a broad antagonistic spectrum against seven tested bacterial and fungal foods pathogens. The 16SDNA sequences showed that the antagonist was *Lactobacillus brevis.* This antimicrobial compound was excreted between late logarithmic and early stationary phases, inactivated by proteolytic enzyme, stable at 120°C and pH 2–6, active in presence of acetone and hexane.

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Key words: *Lactobacillus brevis*, 16S rDNA sequensing, biopreservation, fermented tomato juice.

1. Introduction

In an attempt to prevent growth of pathogens and spoilage microorganisms on produce, research on natural additives has been made. This method is known as biopreservation and consists on extension of the lifetime of food using LAB and/or their metabolites. Food fermentation is a method widely used in the past to extend the storage life of food (De Vuyst and Leroy, 2007; Bourdichon et al., 2012). Numerous studies on fermented food have revealed that they not only have biopreservative properties but also health benefits (Oliveira et al., 2014). Lactic acid bacteria are the major group of bacteria applied in food for improvement of their quality and preservation (Schillinger et al., 1991; Caplice and Fitzgerald, 1999; Deegan et al., 2006; Hsieh et al., 2012). Their health benefits are exerted through several mechanisms including inhibiting the growth of pathogenic bacteria and modifying the host immune response. A number of strains that have been investigated show different properties even between the same species thus emphasizing the importance of strain identification (Sadiq et al., 1989; Holzapfel et al., 1995; Gálvez et al., 2007; Gharsallaoui et al., 2015). Tomato seeds, a major by-product of the tomato processing industry, contain organic acids and phenolic compounds. It is a berry plant in thus there is an appeal and demands of the fruits by the *Solanaceae* family. They can be processed into purées, juices and ketchup. Canned and dried tomatoes are economically important processed products (De Muyncka et al., 2004; Ramos et al., 2013). The aim of this study was to use molecular methods for identify the bacteria responsible to tomatoes fermentation, assess the potential of them to inhibit the growth of bacterial and fungal food pathogens, extract this bioactive substance and investigate their characteristics in foods preservation.

2. Materials and methods

2.1. Tomato juice preparation

Tomatoes *Solanum lycopersicum* L. were collected from Algerian Supermarkets, placed in sterile plastic bags and directly transported to the laboratory. The juice is prepared from the entire pulp, boiling quickly and poured into bottles or jars closed with lids or caps and without added salt or oil or pepper or other flavoring. The bottles were preserved for a period of six months to one year away from light and moisture at 25 °C (Granges et al., 2000).

2.2. Bacterial strain and growth media

One ml of sample from the fermented tomato juice was taken for strain isolation. Sample was diluted with 99 ml of sterile 0.1% peptone water and spiral plated onto MRS-agar and M17-agar media (Oxoid, Ltd., England). MRS plates were incubated at 37°C under both aerobic and anaerobic conditions for 48 h and M17 plates at 44°C under anaerobic condition for 48 h (Rushdy and Gomaa, 2013). All

Gram positive, catalase negative (assayed with 3% v/v H_2O_2) isolates were purified by reisolation and observed under a light microscope. All isolates were coded and stored at - 80°C in either MRS or M17 broth containing equal amounts of 30% sterile glycerol (Oxoid, Ltd., England).

2.3. Screening for potential antimicrobial activity

One ml of the stored isolate was cultured overnight in 20 ml MRS or M17 broth at 37° C, and then 1 ml culture was sub-cultured overnight in 20 ml MRS broth. Cells were removed by centrifuging at 14.000 g for 5 min (Spectrafuge 24D, Labnet, USA). The supernatant was filtered through a sterile 0.22 µm syringe filter (Corning, NY14831, Germany) to obtain a cell free supernatant (CFS). Antimicrobial activity was screened by the well diffusion assay and the spot agar method (Ennahar et al., 2000). *Escherichia coli*, *Salmonella typhi*, *Staphylococcus aureus*, *Clostridium perfringens*, *Pseudomonas aeruginosa*, *Candida albicans*, *Aspergillus flavus*, *Penicillium* sp., and *Fusarium* sp. were used as indicator strains. One ml of each indicator organism (5×10^5 cfu ml^{-1}) was resuspended into 15 ml of MRS-agar (Oxoid, Ltd., England) maintained at 50°C, and immediately poured into a petri dish. After solidification, three wells (5 mm diameter) were cut and 35 µl of a CFS was added to the first well. The remaining CFS was adjusted to pH 6.0 with 1 $mol.l^{-1}$ NaOH in order to rule out possible inhibition effects due to organic acids (Jie Jiang et al., 2012). 35 µl of the pH adjusted CFS was filtered and added to the second well. The neutralized CFS was then treated with 1 $mg.ml^{-1}$ catalase (Oxford Lab. Reagent Co.) at 25°C for 30 min, to eliminate the possibility of H_2O_2 inhibitory action, filtered and placed in the third well. Plates were incubated at 37°C for 48 h. Isolates whose CFS showed inhibition zones in the third well were considered able to produce bacteriocins (De Muyncka et al., 2004) and candidates for selection and further taxonomic identification (Ennahar et al., 2000; De Muynck et al., 2004).

2.4. Genomic DNA extraction, PCR amplification and taxonomical identification

Five pure isolates were selected as potential bacteriocin - producers and submitted to further taxonomical identification procedures. Isolates were grown overnight on MRS-agar plates. Bacterial cells from 3-5 colonies were resuspended in 800 µl of sterile saline solution (NaCl 0.9%, Panreac, Spain) and centrifuged (Spectrafuge 24D, Labnet, USA) for 2 min at 12.000 x g. The supernatant was removed and the cell pellet was submitted to genomic DNA extraction with the InstaGene Matrix (Bio-Rad Laboratories, Spain) according to the manufacturer's instructions. Finally, DNA concentration was measured in a NanoDrop spectrophotometer (Thermo Sci., Spain).

DNA samples were submitted to the PCR reaction based on the rDNA 16S gene (Lane, 1991) for species identification, sequencing the resulting amplification product and subsequent comparison with the sequences of the GenBank data base. Reactions were performed in a T3000 thermocycler (Biometra, Germany), using the primers Uni16 F (5'- AGA GTT TGA TYM TGG CTC AG – 3') and Uni16 R (5'- GGY TAC CTT GTT ACG ACT T – 3') and the recommended amplification program: initial cycle at 95°C for 3 min, followed by 32 cycles at 95°C for 1 min, 52°C for 1 min, and 72°C for 1 min, and a final cycle at 72°C for 7 min and cooling to 8°C (Lane, 1991).

PCR products were analyzed by electrophoresis on 10 g/L agarose gel (D1 low EEO; Pronadisa, Spain) in Tris-borate-EDTA buffer (90 mM Tris-borate, 2 mM EDTA; Panreac, Spain) with the stain Midori Green (0.5 $\mu g.ml^{-1}$; Nippon Genetics, Germany) and visualized under a Chemi Genius UV-lighter (Syngene, United Kingdom) with the GeneSnap 7.12 software (Syngene). Sequencing was performed at the Biomedical Research Center of La Rioja (CIBIR, Lab. of Genomics, Spain). Sequences were assembled with Chromas-Pro 1.49 (Technelysium Pty Ltd., Australia) and analyzed with BLASTn search tool (Altschul et al., 1997; González-Arenzana et al., 2012).

2.5. Antimicrobial compound production

The selected LAB (*Lactobacillus brevis*) was grown in MRS medium. One milliliter of an overnight culture was used to inoculate 100 ml of MRS broth and incubated for 12 h in a static incubator at 37°C. At 2 h intervals, the changes in cell density were recorded at 600 nm. Antimicrobial activity was recorded after adjusting pH to 6.5 by agar diffusion assay (Nowroozi et al., 2004).

2.6. Antimicrobial compound Extraction

The selected LAB strain was propagated in MRS broth at 37°C until the late logarithmic phase and the early stationary phase. For extraction of bacteriocin-like compound, cells were removed by centrifugation (10000 g for 20 min, 4°C), and pH adjusted to 6.5 by means of 1 M NaOH to exclude the antimicrobial effect of the organic acid (Kelly et al., 1996).

2.7. Antimicrobial compound characterization

The inhibiting substances were characterized with respect to pH and thermal stability, susceptibility to denaturation by enzymes, and final treatment with surfactant agents. For pH sensitivity the cell free supernatant (CFS) was adjusted to various pH values in the range of 2–10 with sterile NaOH (3 M) or HCl (3 M) (Sigma Chemical). The pH adjusted samples were incubated at 37°C for 30 min then re-adjusted to pH 6.5 and tested for antimicrobial activities by the spot-on-lawn method. For heat resistance, the CFS

was exposed to heat treatments for 15 min at - 80°C, + 4°C, 80°C, 100°C, and 120°C (Brink et al., 1994; Oh et al., 2000). Enzyme treatments were performed by incubating the CFS in the presence of trypsin (1 mg/ml), amylase (1 $mg.ml^{-1}$), and lipase (1 $mg.ml^{-1}$) (Oxford laboratory reagents) at 37°C for 2 h. After incubation, the enzymes were inactivated by heat treatment at 65°C for 30 min and tested for antimicrobial activity (Oh et al., 2000). Effect of surfactants on antimicrobial activity was tested by adding 1% (w/v) of acetone, chloroform, hexane, and ethanol (Sigma Chemical) to CFS preparations (Todorov et al., 2007). Untreated supernatant and surfactants at each of these concentrations were used as controls. All samples were incubated at 37°C for 5 h and then assayed for antimicrobial activity against indicator strain *Sl. typhi*.

3. Results

3.1. Screening for potential antimicrobial activity

65 strains of bacteria were isolated from traditional fermented tomatoes juice; were tested for their capacity to produce antimicrobial activities against the following indicator microorganisms: *E. coli*, *Sl. typhi*, *S. aureus*, *Cl. perfringens*, *Ps. aeruginosa*, *C. albicans*, *A. flavus*, *Penicillium* sp., and *Fusarium* sp. as shown in Table 1; different profiles of inhibition were observed. Five strains (ISOL01, ISOL02, ISOL03, ISOL04; and ISOL05) were characterized by the broad-spectrum inhibition. The largest spectrum of inhibition was showed by ISOL01 and ISOL03 strains that inhibited seven used indicator microorganisms. Moreover, the active molecule(s) of these strains presents the highest inhibitory effect against all tested microorganisms including the pathogenic Gram-positive bacteria and pathogenic Gram-negative bacteria and the pathogenic fungus A. *flavus*, *Penicillium* sp., and *Fusarium* sp. ISOL02 contained an antimicrobial compound with a limited spectrum that inhibited the growth of five indicators strains, whereas ISOL04 and ISOL05 inhibited only two indicators strains (Tab 1). The results of the antimicrobial assay revealed that the samples demonstrated activity against a number of Gram-positive, Gram-negative bacteria, and fungi.

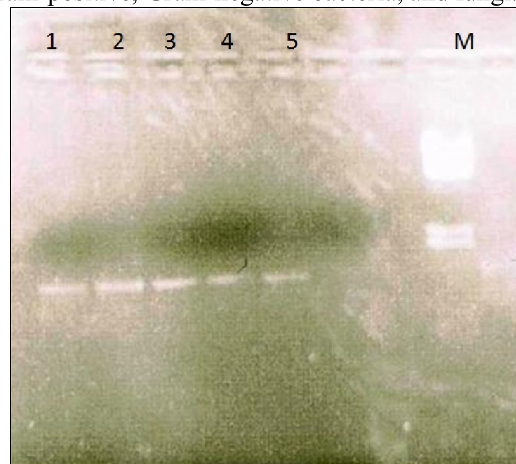

Figure 1. PCR products obtained after amplification of total bacterial DNA with primers specific for lactic acid bacteria. *L. brevis* strains shown as example are lanes 1and 3, *Ps. stutzeri* (lanes 2 and 5), *E. faecium* (lane 4); molecular size DNA marker (M)

Table 1. Antimicrobial activity spectrum of the fermented tomato juice isolates grown on MRS agar for 48 h at 37 °C under aerobic conditions: inhibition zone (mm) exhibited against the indicator bacteria and fungi

Indicator strains	**ISOL01**	**ISOL02**	**ISOL03**	**ISOL04**	**ISOL05**
Escherichia coli	+	-	+	-	+
Salmonella typhi	+	+	+	+	+
Staphylococcus aureus	+	+	+	-	-
Clostridium perfringens	-	-	-	-	-
Pseudomonas aeruginosa	+	-	+	-	-
Candida albicans	-	-	-	-	-
Aspergillus flavus	+	+	+	-	-
Penicillium sp.	+	+	+	+	-
Fusarium sp.	+	+	+	-	-

Legend: +: Inhibitory activity, ISOL: isolate. +: inhibition zone>15mm

3.2. Genotypic identification of strains

Molecular typing of species can help not only to investigate the origin of the strains present in foods but also to establish an association of the various degrees of virulence (López et al., 2006; Foley et al., 2009) or antimicrobial resistance that may exist within a species to certain strains or subtypes. There are plenty of molecular genotyping and subtyping methods. Using the two specific primers: Uni16 F= 5'- AGA GTT TGA TYM TGG CTC AG – 3' 5 (20bases) and Uni16 R= 5'- GGY TAC CTT GTT ACG ACT T – 3'5 (19 bases), we have amplified by PCR a DNA fragment having an expected size of approximately 1300pb. Based on their 16S rDNA

gene sequence similarities (Table 3), the selected strains were identified as *L. brevis* (ISOL01); *L. brevis* (ISOL03), *Ps. stutzeri* (ISOL02), *E. faecium* (ISOL04), *Ps. stutzeri* (ISOL05).

Table 2. DNA concentration of the isolates using NanoVue Plus Spectrophotometer (Nano Drop, Spain)

Samples ID	**ng/µL**	**260/280**	**260/230**	**Const**
ISOL01	450.68	2.26	1.12	50
ISOL02	509.95	2.26	1.12	50
ISOL03	485.45	2.26	1.12	50
ISOL04	579.51	2.22	1.12	50
ISOL05	560.10	2.25	1.12	50

Table 3. Sequence similarity of ISOL01, ISOL02, ISOL03, ISOL04, and ISOL05 strains using 16S rDNA gene sequence PCR and Chromas-Pro 1.49 (Technelysium Pty Ltd., Australia) and analyzed with BLASTn search tool

Isolates	**Primer**	***E. value**	**Significant**	**Sequensing time**	**Description Identity**
ISOL01	Reverse	00	100%	01	*Lactobacillus brevis*
ISOL02	Forward and Reverse	00	100%	03	*Pseudomonas stutzeri*
ISOL03	Forward and Reverse	00	100%	03	*Lactobacillus brevis*
ISOL04	Reverse	00	*Nd	01	*Enterococcus faecium*
ISOL05	Forward and Reverse	00	100%	02	*Pseudomonas stutzeri*

Legend: *E value: is the number of hits one can "expect" to see by chance. The lower the E-value, or the closer it is to zero, the more "significant" the match is." *Nd: Only 1 bad sequence was obtained with the Reverse primer because the PCR fragment was not good. The E value was not 0. We cannot be very sure of this result

3.3. Kinetic of the antimicrobial compound production

The selected strain *L. brevis* started to produce antimicrobial compounds after 6 h incubation in MRS broth at 37°C (Figure 2). Maximum activity was showed after 12 h of incubation (the stationary phase). The maximum OD was also reached after 12 h of incubation. Afterwards, the antibacterial activity slowly decreased.

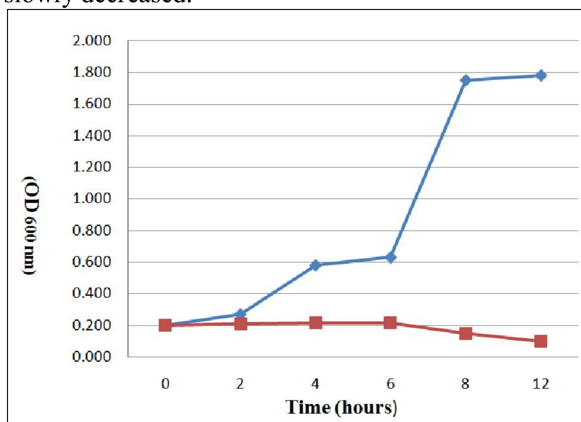

Figure 2 Bacterial growth (◊) and bacteriocin activity of *L. brevis* (□). The strain was grown aerobically in MRS broth at optimal conditions of temperature (37°C), for 12 h

3.4. Antimicrobial compound characterization

Results given in Table 4 showed that the highest antibacterial activity was exhibited in pH 2–6, stable after heat treatment from - 80°C, + 4°C, 80°C, 100°C, and 120°C for 15 min, and it was unstable after treatment with the enzyme trypsin and organic solvents acetone and hexane.

Table 4 Effect of pH, temperature, chemical surfactants and enzymes on the activity of the antimicrobial compounds produced by *L. brevis* against *Sl. typhi*

Treatment	**Bacteriocin activity against *Sl. typhi***
pH	
pH 2.0	+
pH 4.0	+
pH 6.0	+
pH 8.0	-
pH 10.0	-
Temperature	
- 80 °C15 min	+
+ 4°C15 min	+
80°C 15 min	+
100°C 15 min	+
120°C 15 min	+
Surfactants	
Acetone 1 % (w/v)	+
Chloroform 1 % (w/v)	-
Hexane 1 % (w/v)	+
Ethanol 1 % (w/v)	-
Enzymes	
Trypsin (1 mg/ml)	+
Lipase (1 mg/ml)	-
Amylase (1 mg/ml)	-

4. Discussion

Biopreservation is a promising innovative way of extending the shelf-life of fresh fruits and vegetables, and reducing microbial hazards (Settanni and Corsetti, 2008). Tomato is one of the most important vegetables worldwide that need a natural preservation system for extending shelf life, without compromising its quality (Cheigh et al., 2002). Bacteriocins produced by LAB are of great interest to the food fermentation industry because they may inhibit the growth of many food spoilage and pathogenic bacteria (Nowroozi et al., 2004). Therefore, an investigation of bacteriocins-like substance in LAB may offer potential applicability in food preservation (Khay et al., 2011). Therefore, in addition to the broad inhibition spectrum, its technological properties and especially heat and storage stability indicate that the bacteriocin has potential for application as a biopreservative to control pathogens in processed foods (Abo-Amer, 2007).

Bacteriocins produced by *Lactobacillus* spp. have been reported to have a broadly inhibitory effect against several spoilage bacteria (Ogunbanwo et al., 2003a; Ogunshe et al., 2007; Karthikeyan and Santosh, 2009). Screening of potential bacteriocin producers showed that *L. brevis* showed a largest spectrum of inhibition against *E. coli*, *Sl. typhi*, *S. aureus*, *Ps. aeruginosa* (De Muyncka et al., 2004). Tomato seed extracts have shown antimicrobial activity against Gram-positive bacteria and fungi including *S. aureus*, *S. epidermidis*, *Micrococcus luteus*, *E. faecalis*, *B. cereus*, and *C. albicans* (Rabin et al., 2014). *L. brevis* and *Ps. stutzeri* had a high antifungal activity against *A. flavus*, *Penicillium* sp., and *Fusarium* sp. (Smaoui et al., 2010). This was an interesting results for bioconservation of the tomato juice; because fungi cause several potential carcinogenic and mutagenic diseases in humans and animals due to mycotoxin production that are generally thermostable (above 100°C), and thus, can be transferred to food, even after microbial stabilization steps, such as heating and extrusion. Moulds have the ability to grow in a broad range of environmental conditions. It has been estimated that 5-10% of the world's food production is lost because of fungal spoilage (Clark et al., 2012). It is not possible to prevent the introduction of pathogens into food processing facilities, it is crucial to minimize their presence (Akins-Lewenthal, 2012). The most common food preservation strategies applied in the food industry involve chemical or physical techniques. However, these methods only decrease fungal infections and fall short of contaminant elimination.

16S rDNA PCR amplification of selected isolates was performed using universal prokaryotic primers and sequenced. The sequencing results showed that the bacterial isolates from fermented tomato juice were *L. brevis*, *Ps. stutzeri*, and *E. Faecium.* This study showed that the traditional fermented tomatoes evaluated did contain lactic acid bacteria (López et al., 2008; Sadiq et al., 2014). Results from the present work indicated that *L. brevis*, *Ps. stutzeri*, and *E. faecium* were the dominant spices in fermented tomato juice under anaerobic conditions. Different microflora prevails in vegetables especially *Lactococcus* (Kelly et al., 1996), depending upon the type of vegetable, environment and handling procedures. The same results were obtained by Beltrán-Edeza and Hernández-Sánchez, (1989) which found that lactic acid bacteria isolated from tomatoes that were naturally fermented under partial anaerobic conditions were found to be *Leuconostoc mesenteroides*, *L. brevis* and *Streptococcus* sp.

Di Cagno et al. (2009) demonstrated that strains of *L. plantarum*, *Weissella cibaria/confusa*, *L. brevis*, *Pediococcus pentosaceous*, *Lactobacillus* sp. and *E. faecium/faecalis* were identified from raw tomatoes by Biology System. *L. plantarum* was commonly identified in many raw vegetables and, especially, in spontaneous fermented vegetable juices (Buckenhüskes, 1997; Karovičová and Kohajdová, 2003). Occasionally, *Weissella* spp. were identified in vegetables also (Björkroth and Holzapfel, 2006; Di Cagno et al., 2008). *E. faecium* and *L. brevis* are one of bacteria that growth in basic or acidic environments, it can be used in fermenting foods such as cheese and vegetables, and introduced to the starting cultures to inhibit growth of unwanted microbes. *E. faecium* can also be used as a probiotic to out-compete deleterious bacteria in the gastrointestinal tract, and to produces antibacterial peptides called bacteriocins (Kang and Lee, 2005). The presence of *Ps. Stutzeri* in the tomato juice can be explained by it transfer with human, animal, or environmental sources, because *Ps. Stutzeri* are widely distributed in nature due to their high adaptation to various environmental conditions such as food, plants, water, and soil. In soil, the organism has been found in the rhizosphere of cordgrass and other commonly grown plants (Wennan et al., 1998).

Production of our bacteriocin during the early exponential growth phase and the late stationary phase make it in accordance with those of Ogunbanwo et al. (2003b), Rushdy and Gomaa, (2013), and Verellen et al. (1998). Growth beyond the stationary phase resulted in a decrease in bacteriocin-like substance activity. This decrease could be due to the extracellular activity of endogenous proteinases induced during this growth phase. Some reports indicate that bacteriocins are produced throughout the experimental growth phase and not solely during late logarithmic or early stationary phases. The slow decrease of the antibacterial activity in the later

stationary growth phase might be due to the partial digestion of the antibacterial compound by proteolytic enzymes released from the cells (Gong et al., 2010). The stability of bacteriocin activity in acidic pH, heat treatment, enzymes, and in presence of some organic solvents makes them very important for foods biopreservation. Same results were obtained by Abdel-Bar et al. (1987), Rushdy and Gomaa, (2013), and Delves- Broughton et al. (1996).

In general bacteriocins produced by LAB are generally highly stable under acidic conditions, but many of them are easily inactivated under neutral and alkaline conditions. This result could be explained because, at lower pH values (pH 5 and below), the solubility is often increased, less aggregation of hydrophobic peptides occurs, and less bounding of bacteriocins to the cell surface takes place. In addition, hydrophilic compounds may have an enhanced capacity to pass through hydrophilic regions of the cell surface of the sensitive target bacteria (Nettles and Barefoot, 1993; Jack et al., 1995). This heat and pH stability may be useful if the bacteriocin is to be used as an antimicrobial agent in fermented foods or thermally processed foods (Oh et al., 2000).

The bacteriocin produced by *L. brevis* was tested for its sensitivity to various enzymes. It is clear from results that antimicrobial activity was lost or unstable after treatment with trypsinwitch indicated that it was proteinaceous in nature and classified as a bacteriocins-like substance (Olukoya et al., 1993; Jack et al., 1995; Rushdy and Gomaa, 2013).

Finally the consumer trends focus on high-quality, minimally processed green-label foods, thus, driving the food industry towards a focus on natural preservation and the fermentation microorganisms used in food production can antagonize spoilage contaminants, and are increasing in popularity due to their ability to enhance either the product quality, and/or its nutritional profile. Lactic acid bacteria were known to deliver desired technological properties and bioprotection in several different food matrices, concurrently enhancing organoleptic and textural qualities of the final product (Cleveland et al., 2001; Jie Jiang et al., 2012).

Conclusion

The use of bacteriocins and/or bacteriocin-producing strains of LAB are of great interest as they are generally recognized as safe organisms and their antimicrobial products as biopreservatives. However, it is desirable to continue to expand our understanding of the influences that the environments factors have on the implantation of survival bacteriocinogenis strains and the activity of their bacteriocins in order to quantitatively estimate their efficacy for future applications in food model systems.

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